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ON January 28, 2002

Audrey Boyd

January 28, 2002
DATE

Applicant : Dalemans, et al.
Appl. No. : 09/581,976
Filed : June 20, 2000
Title : VACCINE

Grp./A.U. : 1648
Examiner : B. Li

Docket No. : B45124

Assistant Commissioner of Patents
Box AF
Washington, D.C. 20231

DECLARATION OF DR. CATHERINE GÉRARD

1. I, Dr. Catherine Gérard, a citizen of Belgium and residing at 36, Kastanjeboslaan, 1640 Sint Genesius Rhode Belgium, Belgium, declares the following with respect to the invention described and claimed in the patent application 09/581976.

2. I have received the following academic qualifications:

- Bachelor's degree in biology in 1983, Free University of Brussels, Belgium;
- Ph. D. in biological sciences in 1989, Free University of Brussels, Belgium.

I am an employee of GSK Biologicals. I joined SmithKline Beecham Biologicals (the predecessor of GSK Biologicals) in 1996, as a Scientist in the R&D Department. I have been working on several cancer vaccine projects, including HPV-induced lesions and tumors; I am

currently research Senior Scientist heading the preclinical tumor immunology group. I am an inventor of the above case.

3. I have read and am familiar with the Office Action dated August 28, 2001, and with the prior art cited in the Office Action. The prior art does not suggest the need for an improvement of HVP antigenicity.

4. At the time of filing the patent application, it was established that the E6 and E7 genes from HPV 16 were potential antigens to target HPV 16-induced lesions or tumors by immunotherapy (Chen et al., 91). It was also known that these antigens can serve as tumor rejection antigens. CTL raised against E6 or E7 from HPV16 can in certain circumstances have a therapeutic potential on E7 expressing murine tumors. Nothing was known however about whether E6 and E7 proteins of HPV18 could exert similar effects.

5. Furthermore, the best way to induce CTL against these antigens was not known. The publication published in 1997 by Boursnell et al. describes one way to achieve the induction of CTL against E7 proteins using a recombinant vaccinia virus. They describe the generation of the recombinant vaccinia virus coding for mutated, less oncogenic forms of the E6 and E7 antigens. Despite the fact that this recombinant vaccinia virus is shown to induce CTL at least against E7 of HPV16 (there is no demonstration that this is achieved by HPV18-derived proteins), however, the role or functionality of the CTL induced is not demonstrated, let alone in an efficacy tumor model.

The use of proteinD as a fusion partner for E6, E7 and E6E7 fusion is not obvious. The prior art does not suggest either that the increased immunogenicity can be achieved with the combination of such an HPV antigen with a CpG oligonucleotide.

6. In our patent application 09/581976, we describe another, novel, way to induce potent CTL against an early HPV16 antigen, in particular against E7 antigen from HPV16, which is based on the use of a recombinant purified E7 protein produced in *E coli*, fused to helper epitopes provided by a portion of a bacterial protein (PD) and further formulated with CpG ODN used as adjuvant. Moreover, not only do we demonstrate the effectiveness of the CTL induced in an E7 expressing tumor model, but we also show that in addition to CTL we

induced a broader immune response, including CD4 proliferation and although less pronounced, an E7-specific antibody response. These results have been generated with a protein-based approach.

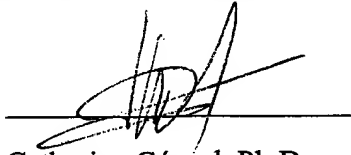
7. The importance of CD4 T cells is now well established in the context of tumor rejection. These cells could either have a direct lytic activity on the tumor, or provide an indirect help to CD8 T cells through the secretion of appropriate cytokines. When an exogenous protein is injected it is generally taken up by antigen presenting cells and presented mainly in the context of MHC class II to CD4 cells. It was not obvious at that time that a vaccine made of a purified protein formulated in an aqueous solution with CpG ODN would lead to the generation of CTL and would lead to tumor rejection. Our data show that it is indeed the case suggesting that the presence of CpG as adjuvant has helped the protein to be delivered into the APC in a different pathway which lead to presentation in Class I HLA and CTL activation.

8. Concomitantly to our experiments, the publication from Chu et al. (published in November 97) confirmed that CpG could work as an adjuvant that switch on TH1 immunity, this, at least when combined to the Hen Egg Lysozyme (HEL) antigen. It was however not obvious at the time this paper was written and at the time we were conducting our experiments that CpG ODN would work with any other antigen, let alone with a cancer antigen.

9. Late antigens from HPV like L1 or L2 are known to form VLP which are immunogenic by themselves and induce antibody responses able to protect against HPV infection.

10. I declare that all statements made herein based on my own knowledge are true and that all statements based on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code, and that such willful false statements may jeopardize the validity of the above application or any patent issued therefrom.

A handwritten signature in black ink, appearing to be 'C. Gérard', is written over a horizontal line.

Catherine Gérard, Ph.D.

Date: January 23, 2002

Human papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen

(tumor immunity/immunosurveillance/immunotherapy/immunoprevention)

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ABSTRACT It has been speculated that immunological mechanisms play an important role in the control of carcinomas associated with human papillomavirus (HPV), such as cervical cancers. We have now demonstrated that immunization of C3H/HeN mice by syngeneic nontumorigenic fibroblast-like cells that contain the transfected HPV-16 E7 gene conferred protection against transplanted cells from a HPV-16 E7-positive syngeneic tumor. This protection was HPV-16 E7-specific and was mediated by CD8⁺ lymphocytes, which presumably were cytotoxic T lymphocytes. These results indicate that tumor cells containing HPV-16 E7, either as a result of transfection, as in our studies, or naturally, as occurs in many human carcinomas, can induce a tumor-specific rejection response and serve as targets for such a response. The system described here provides an animal model to further study immune responses to HPV-associated malignancies and to test the efficacy of anti-HPV vaccines toward the therapy and prevention of such tumors.

The goal of cancer research has been to identify tumor markers that can be used as targets for the selective destruction of neoplastic cells, and it has been hoped since the time of Paul Ehrlich that such markers may be detected in the form of tumor antigens.

The demonstration of tumor-specific transplantation antigen (TSTA) among rodent tumors induced by certain DNA viruses (1, 2) provided much encouragement that this goal may be fulfilled: tumors induced by, e.g., the simian virus 40 (SV40) possess a highly specific TSTA and the expression of this TSTA is closely associated with the neoplastic phenotype (3). However, attempts to find analogous antigens in human neoplasms have failed to yield conclusive information.

Human papillomavirus (HPV) genes and their products have been identified in most cervical carcinomas as well as in other anogenital carcinomas (4, 5). Of the more than 60 types of identified HPVs (6), HPV-16 is one of the types most commonly associated with severe cervical dysplasias and cancers (5, 7). Certain early expressed viral genes and their protein products, especially the E7 nucleoprotein of HPV-16, have been demonstrated to play key roles in both the transformation and maintenance of the malignant phenotype in cell culture systems (8-11). It is of interest that the E7 open reading frame (ORF) encodes a molecule homologous to the SV40 large tumor antigen (12), which is a TSTA expressed by all SV40-induced tumors. Although there has been rapid progress toward the understanding of the molecular biology of HPV-16 and clinical studies have linked certain HPV types to cervical cancers, the roles of host immune responses against HPV-associated tumors remain unclear.

If some HPV oncoproteins could serve as TSTA, efficient immunotherapy may be developed (13). We have, for this reason, introduced the HPV-16 E7 gene into a nontumorigenic, major histocompatibility complex-class I-positive, murine fibroblast line so as to present any putative HPV-16 E7-encoded TSTA as an immunogen to mice, which are then challenged with cells from an HPV-16 E7-positive syngeneic melanoma line. We report here that immunized mice were protected against a challenge with the HPV-16 E7-positive melanoma cells, that this protection was immunologically specific, and that it was mediated by CD8⁺ lymphocytes. We conclude that the HPV-16 E7 gene encodes a TSTA that may provide a highly specific target for immunotherapy and immunoprevention.

MATERIALS AND METHODS

Mice. Female C3H/HeN mice, 6-10 weeks old, were obtained from Charles River Breeding Laboratories.

Cell Lines. The K1735 melanoma line subclone M2 (referred to as "par" cells) (14) and NCTC 2555 fibroblast-cell line (American Type Culture Collection) were of C3H/HeN mouse origin. All cells were maintained at 37°C in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (Sterile System, HyClone), 100 units of penicillin per ml, and 100 µg of streptomycin per ml (referred to as "medium").

Construction of the HPV-16 E7 and E6 Expression Vector pCDM8/E7 and pCDM8/E6. The HPV-16 DNA cloned into pBR322 has been described (15) and was generously provided by L. Gissmann of Deutsches Krebsforschungszentrum, Heidelberg. A 374-base-pair *Taq* I-*Pst* I fragment containing the entire HPV-16 E7 ORF was cleaved from the HPV-16 genome and subcloned into the intermediate plasmids *pic20R* and *pic20H* (16) to introduce a *Hind*III site at the 5' end of the E7 gene so as to insert the E7 gene into *Hind*III-*Pst* I sites in the mammalian expression plasmid pCDM8 (Invitrogen, San Diego). To construct the HPV-16 E6-expressing vector, a *Dde* I fragment containing the entire HPV-16 E6 ORF was subcloned into plasmids pGS62 (17) and *pic20H* (16), then isolated as a *Hind*III-*Xho* I fragment, and ligated into the *Hind*III- and *Xho* I-digested pCDM8 to produce pCDM8/E6 (Fig. 1). Colonies were screened for the described inserts, the appropriate clones were amplified, and their DNA was purified by CsCl/ethidium bromide equilibrium centrifugation.

Transfection. pCDM8/E7 or pCDM8/E6 (20 µg) and PMCIPOLA plasmid (1 µg), which contains the gene encoding neomycin resistance (Stratagene), were cotransfected into par and NCTC 2555 cells by the calcium phosphate

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Abbreviations: CTL, cytotoxic T lymphocyte; HPV, human papillomavirus; mAb, monoclonal antibody; ORF, open reading frame; PCR, polymerase chain reaction; RT, reverse transcription; TSTA, tumor-specific transplantation antigen; SV40, simian virus 40.
*To whom reprint requests should be addressed.

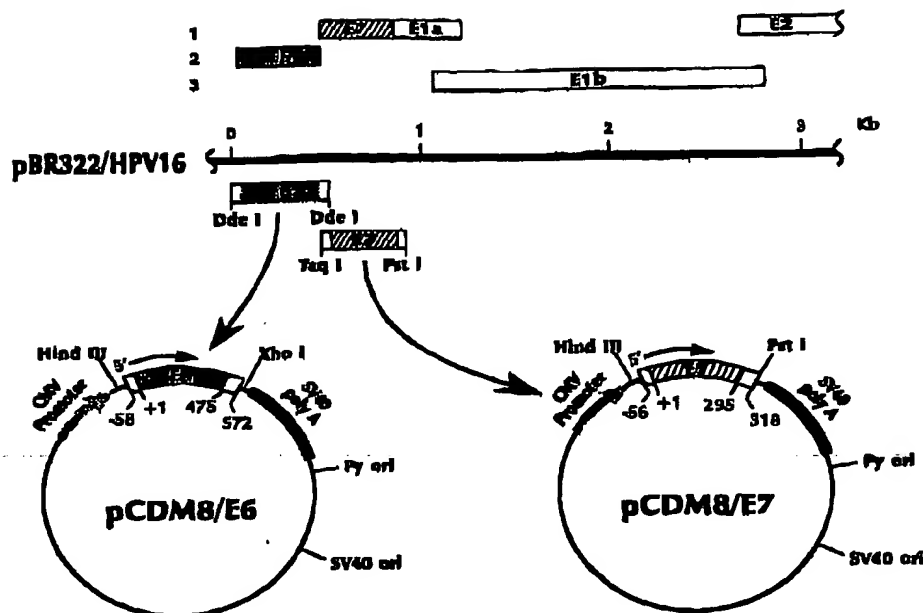


FIG. 1. Construction of the HPV-16 E7-expressing plasmid pCDM8/E7 and the E6-expressing plasmid pCDM8/E6. The entire HPV-16 E7 or E6 ORF was inserted into the HindIII-Pst I or HindIII-Xho I sites, respectively, of pCDM8 plasmids downstream of the cytomegalovirus (CMV) promoter and upstream of the SV40 poly(A)⁺ signal. The figure is not drawn to scale. kb, Kilobase(s).

technique (18). Approximately 48 hr after transfection, the cells were split into a selective medium containing 1 mg of Geneticin (GIBCO) per ml. Ten days later individual clones were picked, expanded, and screened by RNA dot blots. Several E7-positive clones, including one par-derived clone, B7C3, and two NCTC-derived clones, N7.2 and N7.4, were expanded for further characterization. Several E6-positive clones were also expanded. One of these clones, N6.8, was used as a negative control in some experiments.

Nucleic Acid Analysis. Cytoplasmic RNA from individual transfectants was isolated as described (18). Cytoplasmic RNA (1 μ g) was used as template for the amplification reactions. The first-strand cDNA was synthesized by using murine leukemia virus reverse transcriptase (19). The buffer for reverse transcription (RT) contained denatured RNA samples, 1 μ g of denatured random hexamer, all four dNTPs (each at 1 mM), 10 mM sodium pyrophosphate (Boehringer Mannheim), 5 mM dithiothreitol, 10 units of RNasin (Promega), and 18 units of murine leukemia reverse transcriptase (Life Sciences, St. Petersburg, FL) were incubated for 1 hr at 42°C and subsequently at 100°C for 10 min. The supernatants were used for the polymerase chain reaction (PCR). The oligonucleotide primers used for the PCR were HPVA22 (5'-GCATGGAGATACACCTACATTG-3') and HPVA20 (5'-TGGTTTCTGAGAACAGATGG-3') (DNA Factory, San Diego). The cDNA fragments expected to be amplified were 292 base pairs. The PCR mixture from GeneAmp DNA amplification Reagent Kit (Perkin-Elmer/Cetus) contains all four dNTPs (each 200 μ M), 1 μ M primer HPVA22, 1 μ M primer HPVA20, various cDNA synthesized by RT, and 2.5 units of *Thermus aquaticus* DNA polymerase. pCDM8/E7 plasmid (1 ng) was used in the PCR as a positive control. The PCR (denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min) was performed with DNA Thermal Cycler (Perkin-Elmer/Cetus) in 33 cycles. The PCR products were fractionated by electrophoresis on a 1% agarose gel and transferred to nitrocellulose filters. The filter was hybridized under standard con-

ditions (18) with ³²P-labeled DNA fragments containing the E7 ORF. The filters were washed at 68°C, air-dried, and exposed to x-ray film at -70°C.

Tumor Cell Implantation and Measurement of Tumor Growth. Mice, in groups of five, which had either been immunized as indicated or untreated, were each given a single subcutaneous injection on the shaved right sides of the back of 4×10^6 cells from par or HPV-16 E7-transfectant B7C3. Tumor size was assessed by measuring two perpendicular diameters in millimeters by a caliper at regular intervals for each animal. The results were expressed as mean diameter of tumors.

CD8⁺ Cell Depletion *In Vivo*. Mice were injected intraperitoneally with 1.0 ml of ascites fluid diluted in phosphate-buffered saline (PBS) and containing ~ 1 mg of an anti-CD8 monoclonal antibody (mAb) (clone 116-13.1, IgG2a; American Type Culture Collection). As a control, ascites fluid was used which contained isotype-matched anti-CD5 mAb (clone 10.2, kindly provided by L. K. Gilliland). Its concentration of IgG was matched with that of the anti-CD8 ascites fluid, as determined by Paragon serum protein gel electrophoresis (Beckman).

Fluorescence-Activated Cell Sorter Analysis. Single-cell suspensions from spleens were incubated with anti-CD4 (clone GK1.5) or anti-CD8 (clone 53-6) mAb conjugated with fluorescein isothiocyanate (kindly provided by J. A. Ledbetter) at 4°C for 30 min, washed twice with medium, and analyzed on a Coulter Epics C FACS IV, as described (20).

RESULTS

Expression of the HPV-16 E7 Genes in Transfected Murine Cell Lines. To test whether the two murine cell lines, par and NCTC 2555, expressed the HPV-16 E7 gene after transfection with the pCDM8/E7 plasmid, 24 clones from each line were screened by RNA dot-blot assay (data not shown), and three of these clones that gave a positive signal were then examined by radioimmunoprecipitation using specific anti-E7 antise-

rum. We found that E7C3 cells express E7 protein at a level comparable to that seen in CaSki cells, a human cervical cancer cell line. However, the E7 was not detectable above background in N7.2 and N7.4 (data not shown). We further examined expression of E7-specific mRNA in these transfectants. This was done by random-primer extension of cytoplasmic RNA with reverse transcriptase to synthesize first-strand cDNA and by the PCR to amplify the cDNA. The PCR products were then hybridized with 32 P-labeled E7-specific probe in Southern blot analysis. Fig. 2 shows that E7-specific PCR products were detected from three transfectants E7C3 (par origin), N7.2, and N7.4 (NCTC 2555 origin), but not from their parental cell lines. In control experiments, mRNAs were isolated from these transfectants and were pretreated with DNase-free RNase before RT-PCR. No specific PCR product was detected (data not shown), indicating that results shown in Fig. 2 were not due to DNA contamination in cytoplasmic RNA preparations.

Induction of Tumor-Specific Transplantation Immunity to HPV-16 E7. To demonstrate whether transplantation immunity could be induced against an antigen associated with HPV-16 E7-transfectant cells, we injected two NCTC 2555-derived nontumorigenic transfectants, N7.2 and N7.4, intraperitoneally into C3H/HeN mice. These mice were subsequently challenged in the same day subcutaneously by a tumorigenic dose of E7C3 cells or the same amount of par cells. Fig. 3A shows one of five similar experiments. The results demonstrate that mice inoculated intraperitoneally with a control cell line N6.8 (a NCTC 2555-derived clone transfected with the HPV-16 E6 gene in a pCDM8 vector) developed tumors rapidly after E7C3 cell challenge, while all mice inoculated with N7.2 cells developed tumors only transiently. Mice immunized with N7.4 cells also demonstrated significant protection against tumor challenge. Fig. 3B shows that immunization of mice by N7.2 again conferred complete protection against challenge of E7C3 cells. Mice inoculated with PBS or a NCTC-derived transfectant CL19 (provided by M. Kahn), which expresses a human tumor-associated antigen p97 (20, 21), did not display any protection from E7C3 challenge. Fig. 3C shows that the immunization of mice by N7.2 did not confer protection against E7-negative parental K1735 (par) cell challenge. We conclude from these results that immunization of mice with HPV-16 E7 transfectants induces E7-specific transplantation immunity to cells expressing the HPV-16 E7 gene.

CD8⁺ Cells Mediate the Regression of E7C3 Tumor Induced by N7.2 Immunization. Cytotoxic T lymphocytes (CTLs) are central in the removal of virus infected or transformed cells

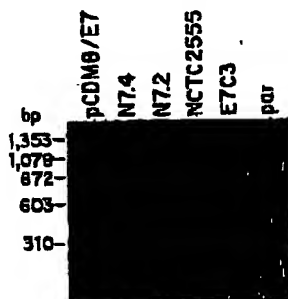


Fig. 2. Southern blot analysis of RT-PCR products from cytoplasmic RNA of HPV-16 E7 transfectants. Lanes: pCDM8/E7, pCDM8/E7 plasmid, amplified by PCR as a positive control; N7.4 and N7.2, NCTC2555-derived HPV-16 E7 transfectants; NCTC2555, the negative control; E7C3, par-derived HPV-16 E7 transfectant; par, negative control. Transfectant mRNA (1 μ g) was used in the RT-PCR and 1 ng of pCDM8/E7 plasmid was used in the PCR. bp, Base pairs.

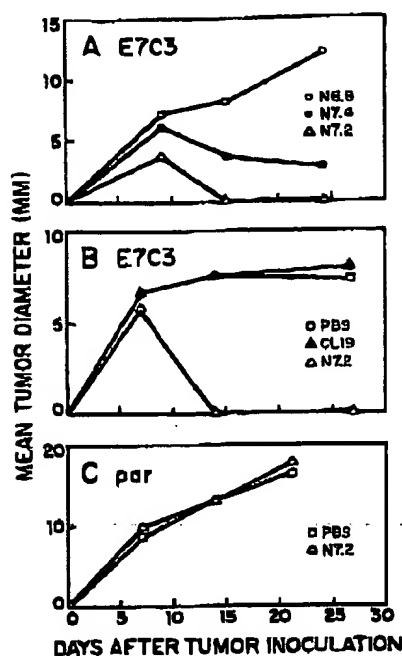


Fig. 3. Growth of murine melanoma cell line E7C3 (par cells transfected with the HPV-16 E7 gene) (A and B) and par cells (E7 negative) (C) in syngeneic C3H/HeN mice immunized with NCTC 2555 fibroblasts that have been transfected with the HPV-16 E7 gene. (A) Groups of five mice were given an intraperitoneal injection of 5×10^6 cells from N6.8 (NCTC2555-derived HPV-16 E6 transfectant) or from either N7.2 or N7.4 (NCTC2555-derived HPV-16 E7 transfectants). (B) Mice were immunized with 5×10^6 N7.2 or CL19 cells (NCTC2555-derived p97 transfectant) or given PBS. In both panels this was followed on the same day by 4×10^6 E7C3 cells transplanted on the right side of the back of mice. (C) Groups of five mice were given an intraperitoneal injection of 5×10^6 par cells transplanted on the right side of the back of mice.

(22). To determine whether lymphocytes expressing the CD8 marker of CTLs are involved in the tumor-specific transplantation immunity induced by immunization with the HPV-16 E7 transfectant, mice that had been immunized by N7.2 cells were treated by an anti-CD8 mAb. Fig. 4 shows one of three similar experiments demonstrating that N7.2-immunized, anti-CD8 mAb-treated mice developed progressive tumors

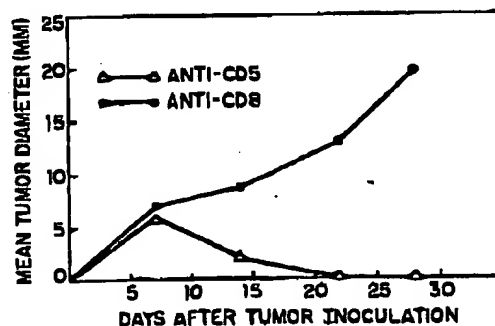


Fig. 4. Effect of anti-CD8 antibody treatment on the tumor growth of mice immunized by N7.2 cells. Groups of five C3H/HeN mice were given an intraperitoneal injection of 5×10^6 N7.2 cells and 1.0 ml of PBS-diluted ascites fluid containing 1 mg of anti-CD8 mAb or, as control, an anti-CD5 mAb. This was followed on the same day by a second injection of 4×10^6 E7C3 cells on the right side of the back of mice.

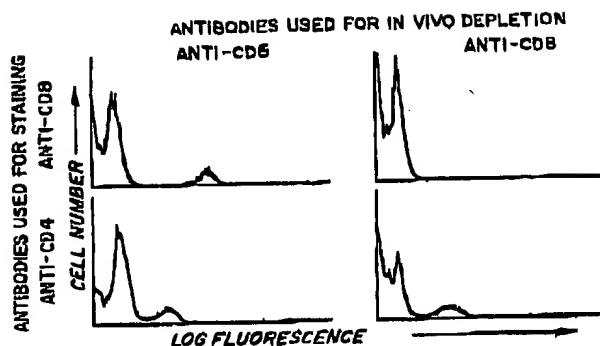


FIG. 5. Flow cytometry analysis of splenocytes from mice treated with anti-CD8 mAb (Right) or mice treated with anti-CD5 mAb (Left). The antibodies were administered intraperitoneally as described in Fig. 4. Splens were removed at day 21 after antibody treatment, and single-cell suspensions were obtained. (Left) Staining profiles of control group splenocytes using fluorescein isothiocyanate-conjugated anti-CD8 and anti-CD4 antibodies. (Right) Staining profiles of splenocytes from anti-CD8-depleted mice are shown. A total of 10^4 cells was analyzed in each panel.

after challenge of a tumorigenic dose of E7C3 cells, whereas N7.2-immunized mice similarly treated by a control (anti-CD5) mAb remained resistant to challenge with E7C3 cells. After injection of the anti-CD8 mAb, the lymphocyte populations were examined to verify depletion of CD8⁺ cells. Fluorescence-activated cell sorter analyses of spleen cells from mice 21 days after treatment with mAb 116-13.1 demonstrate >90% depletion of the CD8⁺ cells as compared to treatment of anti-CD5 control mAb (Fig. 5 Upper). As shown in Fig. 5 (Lower), there was no change in the CD4⁺ subset after treatment with mAb 116-13.1 as compared to mice given the anti-CD5 control mAb. Similar fluorescence-activated cell sorter profiles were obtained at day 5 after mAb injection (data not shown). We conclude that the HPV-16 E7-specific transplantation immunity observed was primarily mediated by CD8⁺ cells and assume that these cells were CTLs.

DISCUSSION

We have demonstrated, in a mouse system, that transfected cells which express the HPV-16 E7 gene can induce a tumor rejection response and serve as its target. Our data further indicate that CD8⁺ T lymphocytes are responsible for the tumor rejection observed.

Nonmalignant NCTC 2555 fibroblasts expressing the HPV-16 E7 gene as a result of transfection were used as the immunogen to confer protection against challenge with HPV-16 E7-transfected K1735 melanoma cells. The response observed cannot be attributed to any antigen shared by NCTC 2555 and K1735 cells because immunization by NCTC 2555-derived clones (CL19) did not protect against E7C3 cell challenge (Fig. 3B) and because immunization by N7.2 cells did not confer protection against challenge with E7-negative K1735 par cells (Fig. 3C). The response must be specific for the HPV-16 E7 antigen because protection against E7-expressing tumor cells E7C3 was observed in mice immunized with cells expressing E7 antigen (N7.2 and N7.4) but not in mice immunized with cells expressing E6 antigen (N6.8) (Fig. 3A). Furthermore, in a preliminary experiment, we found that mice immunized by N6.8 rejected K1735 cells which express HPV-16 E6 (data not shown), but not those expressing HPV-16 E7 (Fig. 3A).

It has been shown that the HPV-16 E7 gene product can transactivate the adenovirus E2 heterologous promoter (23), and the E7 protein regions that are responsible for the

transactivation activity have been mapped by point mutation analysis (24, 25). This raises the possibility that the E7 protein expressed in the transfected cell line may also transactivate other cellular genes whose products could act as targets for tumor rejection or enhance the immune responses to E7-expressing tumor cells.

The most likely assumption is, however, that CD8⁺ T cells in the immunized mice are CTLs and recognize some small fragments from intracellularly processed E7 peptides that are presented at the surface of HPV-16 E7 transfectants in the context of major histocompatibility complex class I molecules (26, 27). Although the H-2K^D class I molecules of C3H/HeN mice can be detected by flow cytometry in the NCTC 2555-derived transfectants, N7.2 and N7.4, they are not detected in cells from the K1735-derived transfectant E7C3 used for challenge (unpublished data). An analogous observation has been made by Tanaka *et al.* (28) who reported that animals immunized by human adenovirus type 12-transformed cells that expressed class I gene as a result of transfection could reject adenovirus type 12-transformed cells that had not been transfected and expressed very low levels of class I antigen. We have observed that the E7C3 cells can be induced to express high levels of class I antigen when treated with interferon (unpublished data) and speculate that interferon or some other cytokines present at the site of transplanted E7C3 cells can upregulate the expression of class I molecules on these tumor cells to a level that makes them accessible to killing by CTLs. We also found that E7 protein could be detected in E7C3, but not in N7.2 and N7.4 cells, in radioimmunoprecipitation experiments (data not shown). This result indicates that E7 protein present at a low level undetectable by radioimmunoprecipitation can still be recognized by CD8⁺ T lymphocytes. A similar result has been reported by Townsend *et al.* (26), who found that the influenza nuclear protein in several L-cell clones that had been transfected with the corresponding gene was not detectable by immunoprecipitation. Nevertheless, these clones could be specifically lysed by nuclear protein-specific cytotoxic T lymphocytes.

Our demonstration that HPV-16 E7-expressing cells can elicit an immune response and that this response can lead to rejection of E7-containing tumor cells suggests that manipulation of a patient's immune system by using, for example, a recombinant vaccine (20, 29) or a purified antigen in adjuvants (30, 31), may make possible the immunotherapy and immunoprevention of human cancers expressing the HPV-16 E7 gene.

We thank Dr. Peter S. Linsley for valuable discussions and William A. Brady and Mitra Singhal for their excellent technical help.

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